CHROM. 4164

A simplified buffer system for gradient elution in amino acid analysis

Despite the availability of amino acid analyzers capable of accelerated chromatograms, many instruments employing the single column, gradient elution procedure of PIEZ AND MORRIS[1] or modifications thereof are in use. These instruments, as exemplified commercially by the Technicon Model NC-1, are particularly advantageous in the analysis of physiological fluids because of their high resolving power. Two disadvantages attendant to their use, however, are (i) the necessity for constructing complex buffer gradients requiring from 14 to 17 individual volumetric additions to the gradient-producing device[1,2] and (ii) the time required for analysis; *viz.* 21 h.

We have designed a simplified and flexible buffer system which requires only two volumetric additions to the gradient-producing device, and have reduced the time for an analysis from 21 to 17 h without sacrificing resolution or precision. These developments are described herein.

Experimental

Instrument. A Technicon Model NC-1 automatic amino acid analyzer (Technicon Corporation, Tarrytown, N.Y.) was used in general accordance with instructions furnished by the manufacturer[2]. Pertinent features of the analyzer as used in this study included: (i) a 140 × 0.636 cm flanged chromatographic column, equipped with a water jacket maintained at 60° and packed to a height of 130 cm with Technicon "Chromobeads, Type B", (ii) a "Micropump" (Technicon) adjusted to deliver eluent buffer to a column at a rate of 0.56 ml/min, and (iii) manifold tubing with nominal pumping rates of 0.45, 0.60, 0.70, and 1.06 ml/min; these were used respectively for column effluent, N_2, ninhydrin reagent, and reactant repump. Buffer gradients were produced in a Technicon nine-chambered Autograd.

Buffers and elution gradients. The composition of the stock buffers is shown in Table I, which also outlines the procedure for constructing the elution gradients used in the modified system and the Technicon system. In producing the standard gradient, all valves in the Autograd were closed while the volumetric additions were made, then all were opened as quickly as possible when the run was started. For the modified gradient, only the first and sixth valves were closed during the construction of the gradient, in which 300 ml of Buffer No. 1 (as modified) was added to any chamber from No. 1 through No. 5 and allowed to reach hydrostatic equilibrium. Similarly, 240 ml of Buffer No. 2 was added to any chamber from No. 6 to No. 9. When the run was started, the first and sixth valves were opened.

Reagents. Ninhydrin reagent was made according to the procedure described by the manufacturer[2], using reagent grade ninhydrin and hydrindantin (Pierce Chemical Co.).

Procedures. The procedures used in the actual analyses were identical to those recommended for 21-h runs with the Technicon Model NC-1 amino acid analyzer[2] except that each chamber of the Autograd was charged with 60 ml, rather than 75 ml, of eluent buffer. In order to obtain maximum utilization of this smaller volume of eluent, the Autograd was tilted during the last hour of the chromatographic run. After the eluent buffer had been exhausted from the Autograd, the system was

TABLE I

PRODUCTION OF BUFFER GRADIENTS

Autograd chamber	*Conventional system*[a]			*Modified system*[b]	
	Buffer No. 1 (pH 2.88)	*Buffer No. 2 (pH 3.80)*	*Buffer No.3 (pH 5.00)*	*Buffer No. 1 (pH 2.88)*	*Buffer No. 2 (pH 5.00)*
1	56 ml + 4.0 ml methanol	—	—		
2	57.6 ml + 2.4 ml methanol	—	—	300 ml (placed anywhere	
3	60 ml	—	—	in chambers 1–5)	
4	60 ml	—	—		
5	—	56 ml	4 ml		
6	4.8 ml	7.2 ml	48 ml		
7	—	—	60 ml		240 ml
8	—	—	60 ml		(placed anywhere
9	—	—	60 ml		in chambers 6–9)

[a] Buffers were prepared as described by manufacturer[2] except Buffer No. 1 was adjusted to a pH of 2.88 rather than 2.875. See text for other minor departures from conventional procedure. All valves closed while filling Autograd.

[b] Buffers were prepared as described[2] except 1 Vol. of ethylene glycol (reagent grade) was admixed with 9 Vol. of Buffer No. 1 prior to use. Only first and sixth valves closed.

switched to an alkaline wash solution (0.2 *N* NaOH) for 30 min, then to a 90-min regeneration cycle with starting buffer.

In separately conducted experiments, continuous recordings were made of the Na^+ concentration and pH of the buffer mixture emerging from the Autograd. Sodium measurements were made with a Technicon Flame Photometer, and pH determinations were made with a Beckman Modular Cuvette (Beckman Instruments, Spinco Division, Palo Alto, Calif.).

Results and discussion

The changes in flow rate and volume of eluent implemented in the present use of the Technicon buffer system permitted a reduction in analysis time from 21 h to about 17 h. It was found advantageous to commence an analysis during the final hour of a normal work day and to terminate it during the first hour of the following work day, thus freeing the operator and the analyzer for other tasks during most of the working day. However, despite efforts to utilize the entire eluent volume, this procedure resulted in the elution of only one-half of the arginine peak; the descending limb of that peak was greatly distorted by the sudden emergence of the alkaline wash fluid. While this difficulty in adapting the Technicon buffer system to a less time-consuming procedure could have been overcome by appropriate increases in eluent volume and flow rate, other difficulties would have resulted. For example, we confirmed the apparent loss of resolution between threonine and serine reported by BOBBITT[3] who used the same gradient at a substantially higher flow rate (0.7 ml/min) to achieve a similar reduction in analysis time.

In contrast to the difficulties mentioned above, the modified buffer system described herein facilitated the desired reduction in instrument time without adversely affecting the resolution of components. As shown by the chromatograms illustrated in Fig. 1, the order of emergence of the ninhydrin-positive compounds was

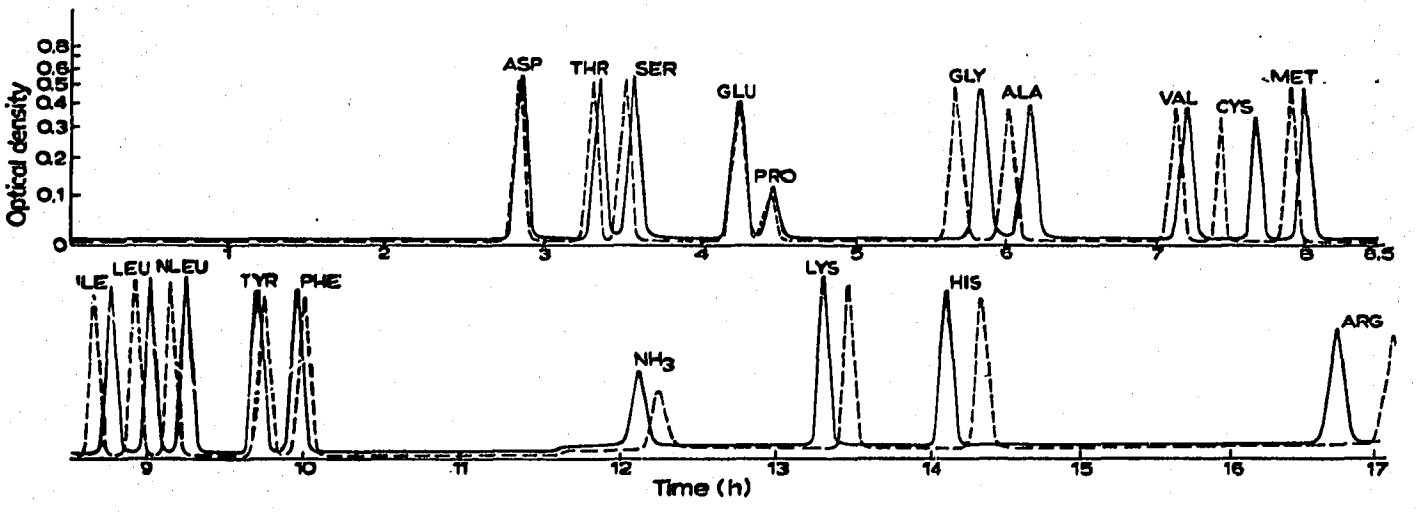

Fig. 1. Chromatograms of a known mixture of amino acids obtained by the modified (——) and conventional (– – – –) buffer gradients. All tracings are based on colorimetric recordings at 570 mμ except for the 440 mμ recording of proline peaks.

the same for both gradients examined in this study. Notwithstanding this similarity, minor differences in the position and shape of certain peaks seemed apparent. With respect to peak positions, use of the modified gradient slightly retarded the elution of all components prior to and including leucine; on the other hand, this gradient accelerated the elution of those compounds emerging after leucine. Differences in peak shape followed a similar pattern; that is, peaks eluted prior to and including leucine with the modified gradient tended to be slightly lower and broader than those eluted

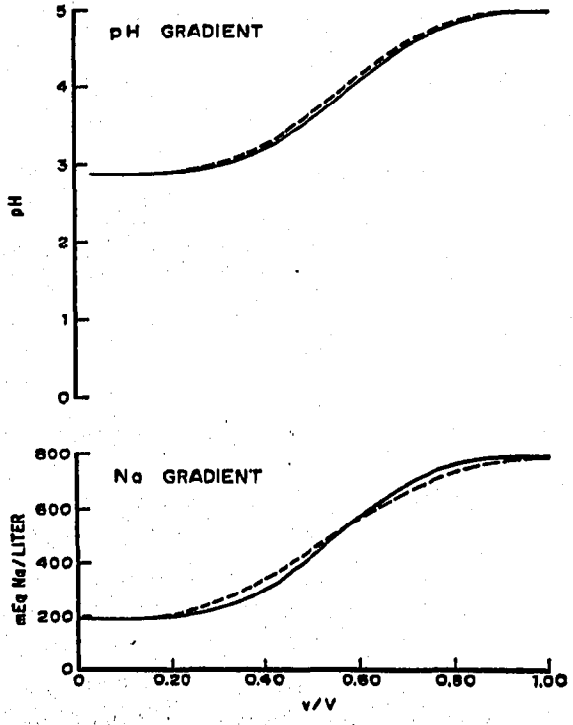

Fig. 2. Sodium and pH gradients produced by the modified (———) and conventional (– – – –) buffer systems: v/V represents progressive emptying of Autograd, where V is the initial volume and v is the volume withdrawn.

with the conventional gradient, whereas those peaks emerging after leucine tended to be narrower and taller with the modified gradient. While not readily apparent from the chromatograms shown in Fig. 1, these differences in peak shape were revealed by a comparison of the height (H) and width (W) values used for estimating peak areas.

The reason for the alterations in the elution times, particularly for arginine, and in peak shapes was sought by determining the pH and Na^+ gradients emerging from the Autograd in each buffer system. The results obtained from these analyses are illustrated in Fig. 2. It is apparent that the pH gradients produced by the two buffer systems were virtually the same. However, the S-shaped curve of the Na^+ gradient produced by the modified buffer system was slightly steeper and its plateau at limit concentration was attained earlier than that of the conventional gradient. These

TABLE II

ANALYSIS OF STANDARD AMINO ACID MIXTURE BY CONVENTIONAL AND MODIFIED BUFFER GRADIENTS

Amino acid[a]	*Gradient*[b]	*Area constant* ($H \times W/\mu mole$)[c]
Aspartic acid	C	49.13 (2.59)
	M	51.77 (1.40)
Threonine	C	51.33 (2.31)
	M	52.93 (1.94)
Serine	C	51.93 (2.12)
	M	53.87 (1.67)
Glutamic acid	C	50.17 (2.76)
	M	50.80 (2.51)
Proline	C	13.20 (1.52)
	M	13.63 (7.35)
Glycine	C	58.27 (0.99)
	M	58.00 (2.15)
Alanine	C	47.53 (2.23)
	M	51.07 (2.16)
Valine	C	45.63 (2.11)
	M	48.63 (2.22)
Half-cystine	C	29.10 (1.57)
	M	29.90 (1.67)
Methionine	C	53.63 (2.59)
	M	53.90 (1.60)
Isoleucine	C	47.27 (2.76)
	M	50.67 (1.68)
Leucine	C	56.83 (2.12)
	M	57.87 (1.93)
Tyrosine	C	55.93 (2.33)
	M	55.93 (1.74)
Phenylalanine	C	55.50 (1.54)
	M	56.00 (1.64)
$(NH_4)_2SO_4$	C	23.90 (7.44)
	M	26.17 (3.98)
Lysine	C	57.30 (3.06)
	M	57.77 (2.14)
Histidine	C	60.17 (2.58)
	M	59.07 (2.13)
Arginine	C	54.17 (3.78)
	M	55.93 (0.88)

[a] Each in a 2.5 mM concentration except cystine, which was 1.25 mM.
[b] C and M denote conventional and modified gradients, respectively.
[c] Values are mean ($n = 3$); coefficient of variation given in parentheses.

findings account for the slightly retarded elution of components emerging before leucine and for the accelerated elution of those in the latter part of the chromatogram. The early plateau of Na^+ is especially noteworthy since it largely explains why arginine was completely eluted by the modified gradient but was only partially eluted by an identical volume of the conventional gradient. Also worthy of special note is the resolution of threonine and serine by the two gradients. While the slightly lower Na^+ concentration in the early part of the modified gradient probably accounted for the slight delay in the elution of these two amino acids, the presence of ethylene glycol in the starting buffer was primarily responsible for the complete separation between the two peaks. The latter conclusion was revealed by the finding that the two peaks did not separate below half-height when distilled water was substituted for ethylene glycol.

The effectiveness of the modified buffer system has been proven by routine laboratory use over about three years time with highly satisfactory results. For the sake of comparison, three consecutive analyses of a standard amino acid mixture by this system and three consecutive runs by the conventional procedure were done under comparable conditions. These results are shown in Table II, which reveals that the two methods are equivalent with respect to reproducibility; moreover, the peak areas are remarkably similar for most of the components. As an overall appraisal, the coefficients of variation for the conventional buffer systems averaged 2.57, and those for the modified system averaged 2.30.

It seems apparent from the foregoing considerations that the modified buffer system described herein substantially reduced the analysis time without sacrificing resolution or precision. The most striking feature of the modified system, however, was its simplicity. Not only was one of the original buffers (pH 3.80) eliminated, but the number of volumetric additions to the Autograd was reduced from 14 to 2; moreover, only two Autograd valves were manipulated. Thus the time, effort, and likelihood for error were substantially reduced.

We are indebted to Mr. JESUS B. GARCIA, JR. for his skilled technical assistance and to Mr. ALTON J. RAHE for statistical analyses.

This work was aided by Grant A-003 of the Robert A. Welch Foundation, Houston, Texas to J. M. PRESCOTT.

Environmental Systems Division, USAF J. P. ELLIS, JR.
School of Aerospace Medicine, Brooks Air Force Base, Texas (U.S.A.)

Department of Biochemistry and Biophysics, J. M. PRESCOTT
Texas A&M University, College Station, Texas (U.S.A.)

1 K. A. PIEZ AND L. MORRIS, *Anal. Biochem.*, 1 (1960) 187.
2 Technicon Corporation, *Amino Acid Analyzer Instruction Manual*, AAA-1, Ardsley, New York, 1967.
3 J. L. BOBBITT, *U.S. Army Med. Res. Lab. (Fort Knox, Ky.)*, Report No. 693, 1966.

Received May 12th, 1969